Reconstitution of the Protein Kinase A Response of the Rat Prolactin Promoter: Differential Effects of Distinct Pit-1 Isoforms and Functional Interaction with Oct-1

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PRL gene transcription is primarily regulated by dopamine, which lowers cAMP levels and inhibits protein kinase A (PKA) activity. Current data indicate that the cAMP/PKA response maps to the most proximal Pit-1/Pit-1β binding site footprint I (FP I) on the rat PRL (rPRL) promoter. Pit-1, a POU-homeo domain transcription factor, is specifically expressed in the anterior pituitary and is required both for the normal development of anterior pituitary cell types, somatotrophs, lactotrophs, and thyrotrophs, and for the expression of their hormones: GH, PRL, and TSHβ. Pit-1 has been shown to functionally interact, via FP I, with several transcription factors, including Oct-1, a ubiquitous homeobox protein, and thyrotroph embryonic factor, which is found in lactotrophs, to activate basal rPRL promoter activity. Pit-1β/GHF-2, a distinct splice isoform of Pit-1, acts to inhibit Ras-activated transcription from the rPRL promoter, which is mediated by a functional interaction between Pit-1 and Ets-1 at the most distal Pit-1 binding site (FP IV). In this manuscript we show 1) that the Pit-1β isoform not only fails to block PKA activation, but is, in fact, a superior mediator of the PKA response; 2) that the PKA response requires intact POU-specific and POU-homeo domains of Pit-1; and 3) that Oct-1, but not thyrotroph embryonic factor, functions as a Pit-1-interacting factor to mediate an optimal PKA response. (Molecular Endocrinology 13: 228–238, 1999)

0888-8809/99/$3.00/0
Molecular Endocrinology

INTRODUCTION

Pit-1 is a POU-homeo domain transcription factor that is specifically expressed in the anterior pituitary, where it is required both for the normal development of three cell types, somatotrophs, lactotrophs, and thyrotrophs (1–3), and for proper expression of the PRL, GH, and TSHβ genes (1, 2). The 33-kDa Pit-1 protein is encoded by six exons (Fig. 1A) that encode a number of important and separable subdomains. On the N terminus two exons make up the 80-amino acid (AA) transactivation domain (TAD), which has been shown to be sufficient for transactivation in mammalian cells (Fig. 1B) (4, 5). On the C terminus, the POU-specific domain (AA 128–198) and POU-homeo domains (AA 213–273) are necessary and sufficient for DNA binding and homodimerization (Fig. 1B) (5–8).

Pit-1β is a splice isoform of Pit-1 that differs only in the β-domain, a 26-AA insertion at position 48 in the TAD caused by the use of an alternate 3′-splice acceptor at the end of the first intron (Fig. 1) (9–11). The AA sequence of the β-domain has been conserved among vertebrates from teleost fish to aves to humans and is present in the predominant Pit-1 isoform found in teleost fish and aves (9–14), suggesting a conservation of function (reviewed in Ref. 15). Moreover, the wild-type AA sequence of the β-domain is required for cell type-dependent display of dominant-negative properties of Pit-1β (15). In pituitary cells Pit-1β represses basal and Ras/Raf-induced rat PRL (rPRL) promoter activity (9–11, 16, 17), while in nonpituitary cells Pit-1β activates basal rPRL promoter activity as well as Pit-1 per unit protein (15). Thus, the sequence conservation of the β-domain, and the distinct transcription properties that it confers upon Pit-1β, sug-

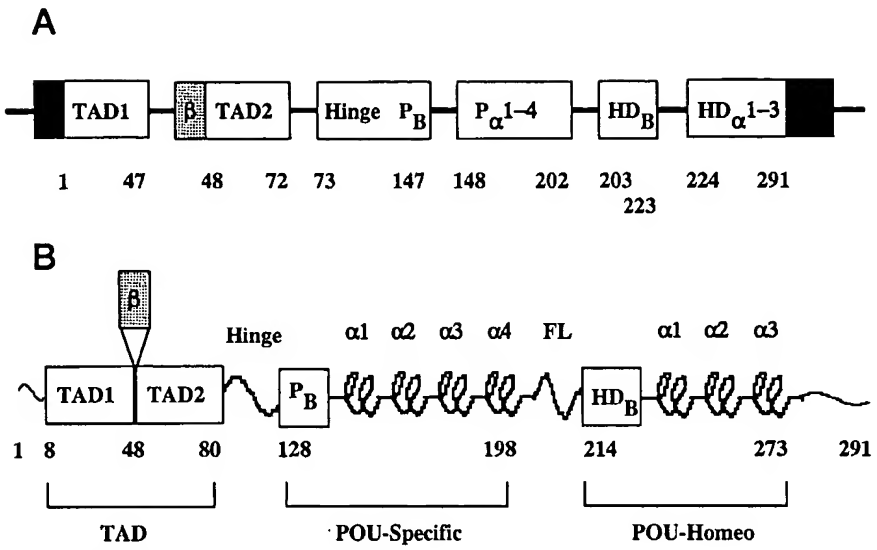

Fig. 1. Genomic and Protein Domain Organization of Pit-1

A, Pit-1 genomic organization. Six exons of Pit-1 with AA endpoints and subdomains they encode are shown as *open rectangles*, 5′- and 3′-untranslated regions (UTRs) as *black rectangles*, and alternatively spliced β-exon as a *gray rectangle*. B, Pit-1/Pit-1β protein domain organization. The functional domains of Pit-1 and Pit-1β proteins, the TAD, the POU-specific, and the POU-homeo domain, are delimited by *brackets* and AA endpoints. Also, P_β and HD_β represent the POU-specific and POU-homeo domain basic domains, and α1–4 and α1–3 represent the POU-specific and POU-homeo domain α-helices. Hinge is the region between the TAD and the bipartite DNA-binding domain, and FL indicates the 15 AA flexible linker between the POU-specific and POU-homeo domain regions.

gest a distinct and conserved role for Pit-1β in the regulation rPRL promoter activity.

The cAMP-protein kinase A (PKA) pathway is important to the regulation of rPRL gene expression in lactotrophs. Dopamine, which lowers cAMP levels and inhibits PKA activity, down-regulates rPRL promoter activity (18–20), while agents such as forskolin, cAMP analogs, or PKA expression vectors, which increase cAMP and activate PKA activity, stimulate rPRL gene transcription in cultured GH4 rat pituitary tumor cells (21–25). Negative regulation by dopamine requires dopamine D2 but not D4 receptors (26) and acts through G(i) α2 to block a positive cAMP and PKA-dependent signaling cascade directed at Pit-1 (19). An additional G(o) α-dependent (and cAMP-independent) pathway may also inhibit PRL gene expression, although that pathway has not been fully characterized (27, 28).

The cAMP-PKA signal is most often transduced by the nuclear transcription factor cAMP-response element-binding protein (CREB), which binds to a specific DNA-regulatory element, the cAMP response element (CRE) (TGACGTCA) (29). However, cAMP-PKA signaling to the rPRL promoter appears to act through a different transducer. The proximal rPRL promoter contains no CRE consensus sites and binds neither affinity-purified CREB from GH_4 cells nor recombinant CREB from bacteria (25, 30). In contrast, cAMP-PKA signaling to the rPRL promoter requires a DNA binding site for Pit-1, footprint I (FP I) (25, 30, 31), and PKA synergizes with Pit-1 to activate rPRL promoter activity in a FP I-dependent manner in a HeLa nonpituitary cell transfection-reconstitution assay developed in this laboratory (31). That Pit-1 is not a direct nuclear target of PKA (32, 33) suggests that a FP I- or Pit-1-associated cofactor is the actual PKA substrate (25, 30, 31, 34).

Pit-1 functionally interacts via FP I with several transcription factors, including Oct-1, a ubiquitous homeobox protein, and thyrotroph embryonic factor (TEF), which is found in lactotrophs, to activate basal rPRL promoter activity (35, 36). These results suggest that Oct-1 or TEF might be the factor that functionally interacts with Pit-1 to mediate PKA signaling through FP I of the rPRL promoter.

To better understand how Pit-1 transduces the PKA signal to the rPRL promoter, we 1) examined whether the Pit-1β isoform differs from Pit-1 as a transducer of the PKA-signal; 2) used deletion mutagenesis to identify domains of Pit-1 required for PKA-signaling; and 3) tested TEF and Oct-1 for ability to function as PKA-signaling cofactors. We show that Pit-1β is a more potent transducer of the PKA response than is Pit-1. Mapping studies with internal deletions of the Pit-1 isoform demonstrate that the POU-specific region of Pit-1 is required for mediation of the PKA response. Further experiments show that Oct-1, but not TEF, acts as a PKA signaling cofactor. These data are important because they show that the PKA signal is mediated by the POU-specific domain, and that Oct-1 can contribute to full reconstitution of the PKA response.

RESULTS

Pit-1β Is a More Potent Enhancer of PKA Signaling Than Is Pit-1

We have previously demonstrated that cAMP/PKA signaling to the rPRL promoter relies not upon the usual transducer, CREB, but rather upon Pit-1 and another factor via FP I of the rPRL promoter (25, 30, 31, 34). Given that the Pit-1β isoform has distinct effects on basal and Ras-activated transcription (9–11, 16, 17), it seemed possible that Pit-1β might also display altered ability to transduce PKA signaling. To address this issue, we examined the relative abilities of Pit-1 and Pit-1β to stimulate basal and PKA-stimulated rPRL promoter activity in a HeLa nonpituitary cell transfection-reconstitution assay developed in this laboratory (31). First, both isoforms were tested for activation of basal rPRL promoter activity. Increasing, but low and nonsaturating, doses of pRSV-Pit-1 and pRSV-Pit-1β were introduced into HeLa nonpituitary cells by electroporation with a rPRL-driven luciferase reporter (pA3–425 rPRL Luc) and assessed for basal transcriptional potency (Fig. 2). The rPRL-driven luciferase reporter alone produced about 5 relative light units (RLU), and pRSV Pit-1 DNA doses of 0.03, 0.3, and 3 μg increased rPRL promoter activity to 1.4-, 1.9- and 11.9-fold, respectively (Fig. 2A). Identical pRSV-Pit-1β DNA doses had no discernible effect upon rPRL promoter activity. These findings are consistent with earlier work in which the lower levels of Pit-1β protein, expressed per unit DNA (see also Fig. 2C), result in lower activation of basal rPRL promoter activity in pituitary cells (10, 15).

To assess the relative abilities of Pit-1 and Pit-1β to enhance stimulation of rPRL activity by PKA, the same doses of pRSV-Pit-1 and pRSV-Pit-1β were introduced into HeLa nonpituitary cells by electroporation with pA3–425 rPRL Luc and pRSV-PKAβ, a plasmid encoding the catalytic β-subunit of PKA (Fig. 2B). While PKAβ alone increased rPRL promoter activity 35-fold, the addition of 0.03, 0.3, and 3 μg DNA doses of pRSV-Pit-1 further increased promoter activity to 164-, 306- and 148-fold, respectively (Fig. 2B). Identical DNA doses of pRSV-Pit-1β increased promoter activity to 73-, 344-, and 535-fold, respectively. Surprisingly, the highest dose of Pit-1 in the presence of PKA had a smaller effect on promoter activity than did the two lower doses (Fig. 2B). Moreover, the maximal fold induction was greater for Pit-1β/PKAβ (535-fold) than for Pit-1/PKAβ (306-fold). The blunting of the response of the rPRL promoter at high Pit-1 doses in the presence of PKAβ is a novel observation. Indeed, previous reports have shown that increasing Pit-1 DNA input results in a consistent dose-dependent increase in basal rPRL promoter activity, even at 10-fold higher Pit-1 DNA inputs (15). Furthermore, no such blunting of the response to Pit-1β by PKAβ has been observed, at these or even at 10-fold higher Pit-1β DNA doses (15). These two points indicate that the blunting of the PKA response at the highest Pit-1 DNA dose is not simply due to squelching.

It is possible that PKAβ might affect the transcriptional potency of Pit-1 or Pit-1β by altering Pit-1/Pit-1β protein expression. To rule this out, we performed Western blot analysis on extracts from HeLa cells transfected with combinations of Pit-1, Pit-1β, and PKAβ. We first attempted to visualize Pit-1 isoforms with a rabbit polyclonal anti-Pit-1 antibody, but found that it lacked sufficient sensitivity to detect Pit-1β protein. To increase the sensitivity of the Western blot assay, Pit-1 and Pit-1β were N-terminally tagged with the influenza hemagglutinin (HA) epitope, to allow use of the more sensitive monoclonal anti-HA antibody. The HA tag has no effect on the ability of Pit-1 or Pit-1β to activate rPRL promoter, alone or with known cofactors (15). HA-Pit-1 and HA-Pit-1β were not detected in mock-transfected HeLa cells (Fig. 2C, lane 1) or in HeLa cells transfected with pRSV-PKAβ only (Fig. 2C, lane 2). Easily detectable and constant levels of HA-tagged Pit-1 protein were found in HeLa cells transfected with pRSV-HA-Pit-1 without or with pRSV PKAβ (Fig. 2C, lanes 3 and 4), and lower, but still constant, levels of HA-tagged Pit-1β protein were detected in HeLa cells transfected with pRSV-HA-Pit-1β without or with pRSV PKAβ (Fig. 2C, lanes 5 and 6). The blot was reprobed with a monoclonal antiactin antibody to demonstrate that all lanes were loaded with equal amounts of cell lysate (Fig. 2C). These data demonstrate that while Pit-1β protein is indeed expressed at a lower level per unit input DNA than is Pit-1, the addition of PKAβ does not affect these protein expression levels. Taken together, the data presented in Fig. 2 reveal that Pit-1β is a superior transducer of PKA signaling, reaching levels of induction above those reached by Pit-1 at any DNA dose. Moreover, the observation that Pit-1β protein is expressed at lower levels per unit DNA input than is Pit-1 would indicate that the PKAβ response may be underestimated for this isoform, since the PKAβ-fold was not normalized to the amount of HA Pit-1 protein expressed.

This laboratory has shown that it is, in fact, the AA sequence of the β-domain that determines the unique properties of Pit-1β (15). Here we have shown that Pit-1β is a superior transducer of the PKA signal and thus implicated the β-domain as one domain of Pit-1 intrinsic PKA signal-transducing properties. However, the ability of the Pit-1 isoform to transduce the PKA signal means that there are other domain(s), shared by both isoforms, with intrinsic PKA-modulating properties. For example, the TAD alone is sufficient, when fused to the LexA DNA-binding domain (DBD), to mediate the dopamine repression of a target promoter through a reduction of PKA signaling (37).

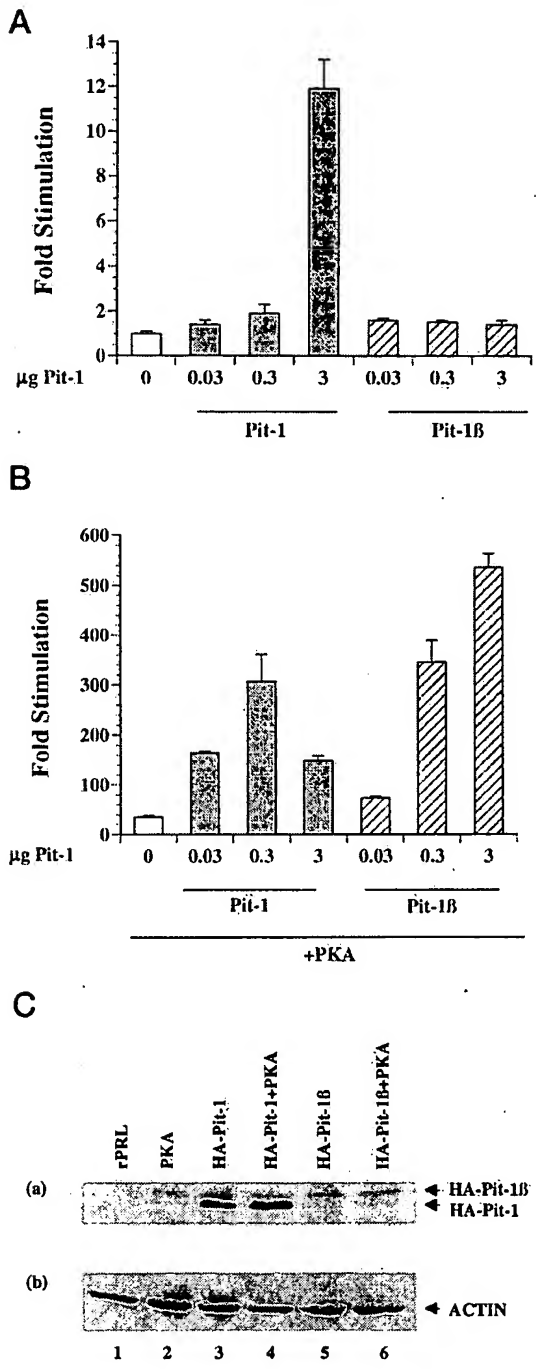

Fig. 2. Pit-1β Is a Superior Mediator of PKA Signaling

For panels A and B, combinations of 3 μg pA3 -425 rPRL luc and either pRSV-Pit-1 (0.03, 0.3, or 3 μg), pRSV-Pit-1β (0.03, 0.3, or 3 μg), and 10 μg pRSV-PKAβ were introduced into HeLa nonpituitary cells by electroporation. pRSVβ-globin was added to keep the total amount of RSV promoter-containing DNA constant. Cells were harvested after 44–48 h and total light units measured, as described in *Materials and Methods*. Results for panels A and B are expressed as the mean ± SD of a representative experiment of three experiments done in duplicate. A, Effects of increasing DNA doses of Pit-1- *vs.* Pit-1β-expressing plasmids on basal rPRL promoter activity. B, Effects of increasing DNA doses of Pit-1- *vs.* Pit-1β-expressing plasmids on PKA-stimulated rPRL promoter activity. C, Analysis of Pit-1 isoform expression ± PKA. Combinations of pA3PRLluc-425, pRSV-HA-Pit-1, pRSV-HA-Pit-1β, and PKAβ were introduced into HeLa nonpituitary cells by electroporation. pRSVβ-globin was added to keep the total amount of RSV promoter-containing DNA constant. Lanes were loaded with equal protein (100 μg) from extracts of HeLa cells transfected as follows: 3 μg of pA3PRLluc-425 (lane 1); 3 μg of pA3PRLluc-425 and 10 μg of pRSV-PKA (lane 2); 3 μg of pA3PRLluc-425 and 10 μg of pRSV-HA-GHF1 (lane 3); 3 μg of pA3PRLluc-425, 10 μg of pRSV-PKA, and 10 μg of pRSV-HA-GHF1 (lane 4); 3 μg of pA3PRLluc-425 and 10 μg of pRSV-GHF2 (lane 5); 3 μg of pA3PRLluc-425, 10 μg of pRSV-PKA, and 10 μg of pRSV-GHF2 (lane 6). After 24 h, cells were harvested and analyzed by SDS-PAGE. (a) The blot was probed with a mouse monoclonal anti-HA antibody. BSA-HA was used as a positive control (data not shown). (b) To demonstrate equal protein loading, the blot was stripped and probed with a mouse monoclonal antiactin antibody.

The POU-Specific and POU-Homeo Domains of Pit-1 Are Required for PKA Signaling

To identify the shared region(s) of Pit-1 and Pit-1β that are required for efficient transduction of the PKA signal to the rPRL promoter, wild-type and internally deleted versions of Pit-1 were introduced into HeLa nonpituitary cells by electroporation in the presence or absence of PKAβ and assessed for basal transcriptional potency, for the ability to mediate PKA signaling (Fig. 3A), and for protein expression level (Fig. 3B). Specific DNA doses that result in equivalent Pit-1 protein levels were determined for each construct, as in previous studies (Fig. 3B) (15). However, four constructs, Δ 2–45, Δ 48–73, Δ 209–252, and Δ 255–291, failed to yield equivalent protein expression regardless of DNA input.

In Fig. 3A, the basal transcription potency of each internally deleted Pit-1 construct is shown. The basal transcription potency of full-length Pit-1, which activated rPRL promoter activity 6-fold, was normalized to 100% (Fig. 3A). Two internal deletion constructs, Δ 2–45 and Δ 48–73, which remove portions of the TAD, displayed 3-fold reductions in transcription potency. However, under these experimental conditions, their levels of protein expression were also lower than wild type (Fig. 3B). Internal deletion construct Δ 72–125, which removes the hinge region between the TAD and POU-specific domain, displayed a greater than 10-fold increase in basal transcription potency, but also a higher level of protein expression. Two internal deletion constructs, Δ 124–201 and Δ 178–201, which remove portions of the POU-specific domain, displayed 2- to 3-fold reductions in basal transcription potency, yet were expressed at higher protein levels than wild type. Also, internal deletion Δ 200–211, which deletes a region between the POU-specific and POU-homeo domains, displayed basal transcription po-

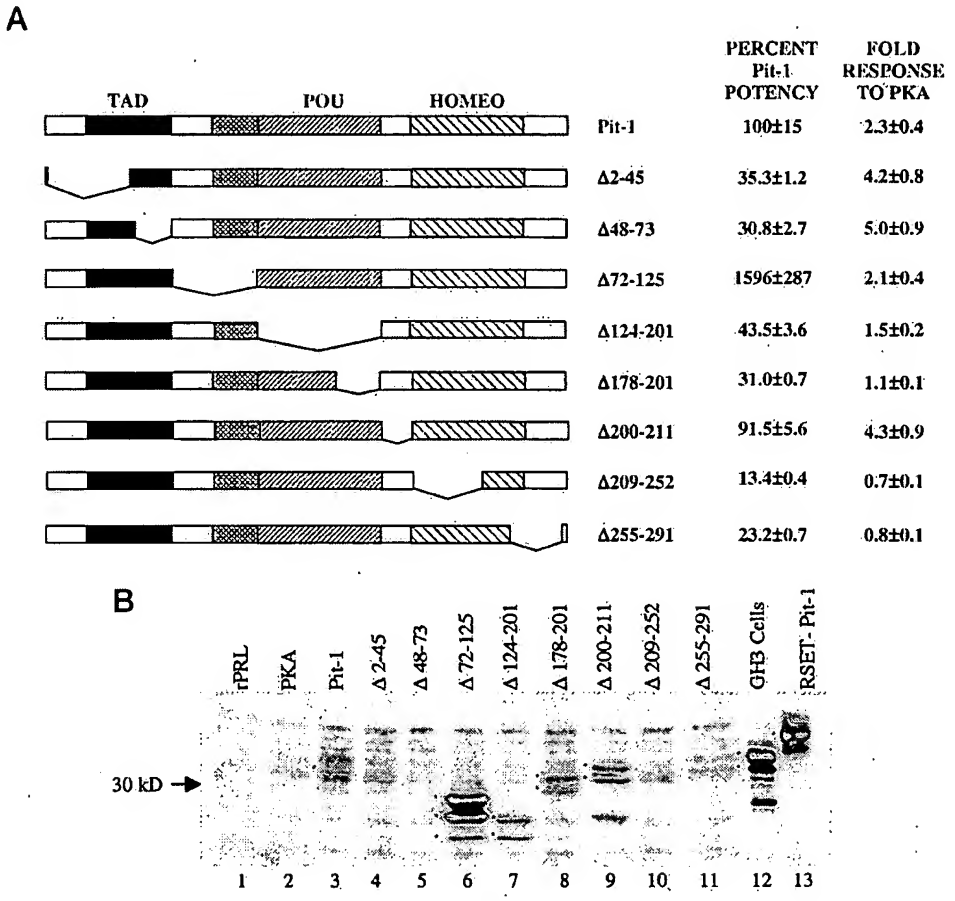

Fig. 3. Deletion Mapping of Functionally Important Regions of Pit-1

A, Effects of internal deletions on Pit-1 transcription potency. Combinations of pA3PRLluc-425 (3 μg), pRSV-PKAβ (10 μg), Pit-1 (3 μg), Pit-1Δ2–45 (15 μg), Pit-1Δ48–73 (15 μg), Pit-1Δ72–125 (2 μg), Pit-1Δ124–201 (3 μg), Pit-1Δ178–201 (10 μg), Pit-1Δ200–211 (3 μg), Pit-1Δ209–252 (10 μg), and Pit-1Δ255–291 (10 μg) were introduced into HeLa nonpituitary cells by electroporation. Specific DNA doses that result in essentially equivalent Pit-1 protein levels were determined for each construct as in previous studies (15). pRSVβ-globin was added to keep the total amount of RSV promoter-containing DNA constant. Cells were harvested after 48 h, total light units measured, and Pit-1 potency expressed as percent of wild-type (100% = 6-fold); PKA response was calculated as described previously. The domain structure of Pit-1 is shown at the *top*. The TAD is represented by the *solid black box*; the DNA-binding domains, the POU-specific (POU), and the POU-homeo (HOMEO) are repesented by the *hatched boxes*, and a region with several negatively charged residues is shown by the *checked box*. The regions deleted by the various Pit-1 internal deletions are indicated in the *schematic diagram*, and the AA end points of the deleted areas are shown to the *right*. These data are expressed as the mean ± SD of a representative experiment of three experiments done in duplicate. B, Western analysis of Pit-1 deletion mutants expressed in HeLa cells. Lanes were loaded with equal protein (100 μg) from extracts of HeLa cells transfected as follows: 3 μg of pA3PRLluc-425 (lane 1); 10 μg of pRSV-PKAβ (lane 2); 3 μg of pRSV-Pit-1 (lane 3); 15 μg of Pit-1Δ2–45 (lane 4); 15 μg of Pit-1Δ48–73 (lane 5); 2 μg of Pit-1Δ72–125 (lane 6); 3 μg of Pit-1Δ124–201 (lane 7); 10 μg of Pit-1Δ178–201 (lane 8); 3 μg of Pit-1Δ200–211 (lane 9); 10 μg of Pit-1Δ209–252 (lane 10); 10 μg of Pit-1Δ255–291 (lane 11); GH3 pituitary cell extract (lane 12); and RSET-Pit-1 (a bacterially produced Histidine-tagged version of Pit-1) (lane 13). The blot was probed with a rabbit polyclonal anti-Pit-1 antibody specific for AA 36–52 and 214–230 (BAbCO).

tency similar to wild type, but was expressed at higher levels than wild type. Two internal deletion constructs, Δ 209–252 and Δ 255–291, which remove portions of the POU-homeo domain, displayed lower levels of basal transcription potency and protein expression. Because differences of basal transcription potency not corresponding to equivalent differences of protein expression level are meaningful, the negative effects of internal deletion constructs, Δ 124–201 and Δ 178–201, which remove the POU-specific domain, and Δ 200–211, which removes a region between the POU-specific and POU-homeo domains, on basal transcription potency are significant. The negative effects of the POU-homeo domain deletions, Δ 209–252 and Δ255–291, while accompanied by a decreased protein expression level, may also be due, in part, to loss of DNA-binding function of the homeo domain.

Nevertheless, the pattern of basal expression by these deletion constructs is consistent with that noted previously (4).

The PKA responsiveness of each internally deleted Pit-1 construct is shown in Fig. 3A. This responsiveness is derived from the ratio of fold activation with and without PKAβ and, thus, takes into account the protein expression level of each construct. PKAβ increased the transcription potency of full-length Pit-1 by about 2-fold. The TAD-specific internal deletion constructs, Δ2–45 and Δ48–73, displayed an increased response to PKA (4- to 5-fold). The hinge-specific internal deletion construct, Δ72–125, was as responsive as wild type to PKA. The two POU-specific internal deletion constructs, Δ 124–201 and Δ 178–201, were insensitive to PKA (1.5- and 1.1-fold, respectively), while the Δ 200–211 construct was more responsive to PKA than wild type (4.3-fold). The two POU-homeo domain-specific internal deletion constructs, Δ 209–252 and Δ 255–291, were relatively insensitive to PKA signaling. Because the level of protein expression of Pit-1 is not affected by PKA (Fig. 2D), any decreased sensitivity to PKAβ is significant. Thus, loss of the C terminus (or all) of the POU-specific domain reduces the ability of Pit-1 to transduce the PKA signal, as does loss of the POU-homeo domain. Of note, deletion of either half of the TAD or of the region between the POU-specific and POU-homeo domains increased responsiveness to PKA signaling.

TEF Is Not the Pit-1/PKA Coactivator

Pit-1, although an important transducer of PKA signaling, is not a direct nuclear target of PKA (32, 33). Thus, a FP I- or Pit-1-associated coactivator is likely to be the actual substrate of PKA (25, 30, 31, 34). TEF and Oct-1, both of which bind to FP I (35, 36), seemed likely candidates for a PKA-signaling coactivator.

To determine whether TEF acts as a Pit-1 coactivator to transduce the PKA signal to FP I, combinations of pRSV-Pit-1, pRSV-Pit-1β, pRSV-FLAG-TEF, and pRSV PKAβ were introduced into HeLa nonpituitary cells by electroporation and assessed for the ability to mediate PKA signaling (Fig. 4A). In these experiments, PKAβ alone stimulated rPRL promoter activity 5-fold. TEF further increased the stimulation of rPRL promoter activity to 17-fold. Pit-1 and Pit-1β increased rPRL promoter activity to 33- and 96-fold, respectively. TEF and either Pit-1 or Pit-1β worked in a merely additive manner; the combination of TEF and Pit-1 stimulated promoter activity to 79-fold, and the combination of TEF and Pit-1β stimulated promoter activity to 163-fold. Western blot analysis of HeLa cell extracts with a mouse monoclonal anti-FLAG antibody shows that neither PKA nor Pit-1 or Pit-1β alter the levels of FLAG-tagged TEF expression (Fig. 4B). These data show no evidence of synergy between TEF and Pit-1 or Pit-1β in mediating the PKA signal and thus do not support a role for TEF as a Pit-1/PKA coactivator.

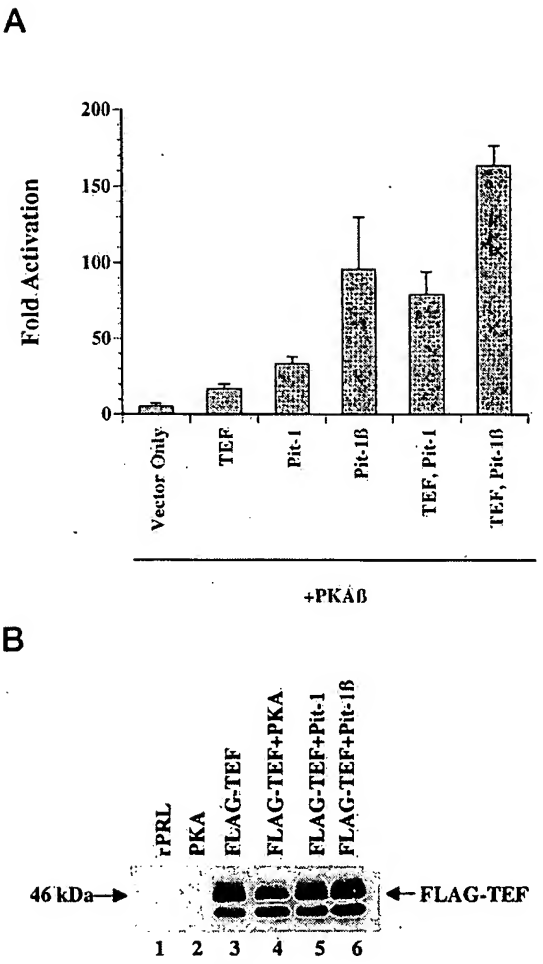

Fig. 4. TEF Has Little Effect on the PKA Response

A, Effects of TEF on PKA-activated rPRL promoter activity in HeLa. Plasmid pA3PRLluc-425 (3 μg) and combinations of pRSV-PKAβ (10 μg), pCMV-FLAG-hTEF (0.05 μg), pRSV-Pit-1 (3 μg), and pRSV-Pit-1β (3 μg) were introduced into HeLa nonpituitary cells and harvested as described in *Materials and Methods*. Fold activation of basal rPRL promoter activity was calculated by dividing the mean RLU obtained in the presence of exogenous transcription factors by the mean RLU obtained in the absence of exogenous transcription factors. These data represent the mean ± SD of two experiments performed in triplicate. B, Western analysis of TEF expression in HeLa cells. Lanes were loaded with equal protein (100 μg) from extracts of HeLa cells transfected as follows: 3 μg of pA3PRLluc-425 (lane 1); 3 μg of pA3PRLluc-425 and 10 μg of pRSV-PKAβ (lane 2); 3 μg of pA3PRLluc-425 and 3 μg of pCMV-FLAG-hTEF (lane 3); 3 μg of pA3PRLluc-425, 3 μg of pCMV-FLAG-hTEF, and 10 μg of pRSV-PKAβ (lane 4); 3 μg of pA3PRLluc-425, 3 μg of pCMV-FLAG-hTEF, and 3 μg of pRSV-Pit-1 (lane 5); 3 μg of pA3PRLluc-425, 3 μg of pCMV-FLAG-hTEF, and 3 μg of pRSV-Pit-1β (lane 6). Total plasmid amount was maintained constant with pRSVβ-globin DNA. The blot was probed with mouse monoclonal anti-FLAG antibody as described in *Materials and Methods*.

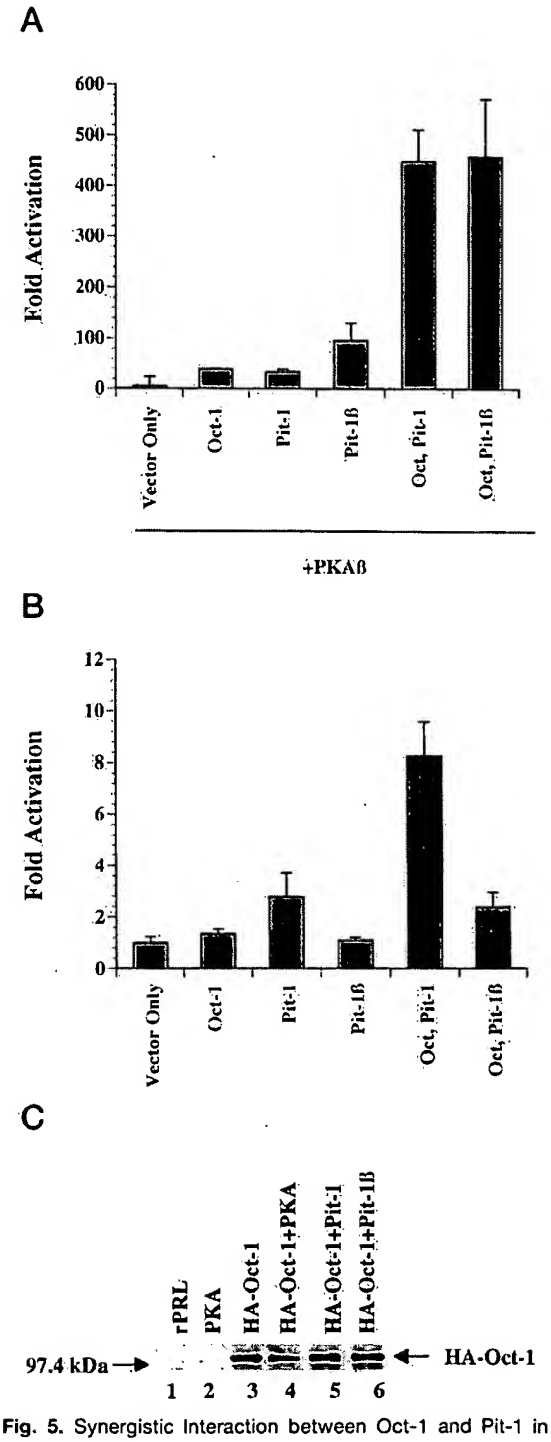

Fig. 5. Synergistic Interaction between Oct-1 and Pit-1 in PKA Signaling

A, Effects of Oct-1 on basal rPRL promoter activity in HeLa cells. Plasmid pA3PRLluc-425 (3 μg) and combinations of pCGN-Oct-1 (0.05 μg), pRSV-Pit-1 (3 μg), and pRSV-Pit-1β (3 μg) were introduced into HeLa nonpituitary cells and harvested as described in *Materials and Methods*. Fold activation of basal rPRL promoter activity was calculated by dividing the mean RLU obtained in the presence of exogenous transcription factors by the mean RLU obtained in the absence of exogenous transcription factors. These data represent the mean ± SD of two experiments performed in triplicate. B, Effects of Oct-1 on PKA-activated rPRL promoter activity in HeLa cells. Plasmid pA3PRLluc-425 (3 μg) and combinations of pRSV-PKAβ (10 μg), pCGN-Oct-1 (0.05 μg), pRSV-Pit-1 (3 μg), and pRSV-Pit-1β (3 μg) were introduced into HeLa nonpituitary cells by electroporation. Fold activation of basal rPRL promoter activity was calculated by dividing the mean RLU obtained in the presence of PKA by the mean RLU obtained in the absence of PKA. C, Western analysis of Oct-1 expression in HeLa cells. Lanes were loaded with equal protein (100 μg) from extracts of HeLa cells transfected as follows: 3 μg of pA3PRLluc-425 (lane 1); 3 μg of pA3PRLluc-425 and 10 μg of pRSV-PKAβ (lane 2); 3 μg of pA3PRLluc-425 and 3 μg of pCGN-HA-Oct-1 (lane 3); 3 μg of pA3PRLluc-425, 3 μg of pCGN-HA-Oct-1, and 10 μg of pRSV-PKAβ (lane 4); 3 μg of pA3PRLluc-425, 3 μg of pCGN-HA-Oct-1, and 3 μg of pRSV-Pit-1 (lane 5); 3 μg of pA3PRLluc-425, 3 μg of pCGN-HA-Oct-1, and 3 μg of pRSV-Pit-1β (lane 6). Total plasmid amount was maintained constant with pRSVβ-globin DNA. The blot was probed with mouse monoclonal anti-HA antibody as described in *Materials and Methods*.

Oct-1 Is a Pit-1/PKA Coactivator

To determine whether Oct-1 acts as a Pit-1 coactivator to transduce the PKA signal to FP I, combinations of pRSV-Pit-1, pRSV-Pit-1β, pRSV-HA-Oct-1, and pRSV-PKAβ were introduced into HeLa nonpituitary cells by electroporation with pA3–425 rPRL Luc and assessed for ability to mediate PKA signaling (Fig. 5A) and basal transcriptional potency (Fig. 5B). In these experiments, PKAβ alone stimulated rPRL promoter activity 5-fold. Oct-1 further increased the stimulation of rPRL promoter activity to 38-fold. Pit-1 and Pit-1β increased rPRL promoter activity to 33- and 96-fold, respectively. Oct-1 and either Pit-1 or Pit-1β worked in a synergistic manner; the combination of Oct-1 and Pit-1 stimulated promoter activity to 449-fold, and the combination of Oct-1 and Pit-1 stimulated promoter activity to 458-fold.

By contrast, without PKA, Oct-1 synergized with Pit-1 and Pit-1β in a much more modest manner. Oct-1 alone increased basal rPRL promoter activity 1.5-fold (Fig. 5B). Pit-1 increased rPRL promoter activity 3-fold, while Pit-1β had little effect on promoter activity. Oct-1 and Pit-1 or Pit-1β synergized to 8- and 2.5-fold, respectively. These results are consistent with previous data showing that Oct-1 and Pit-1 synergistically activate rPRL promoter activity (38). However, we see a much stronger synergy in the presence of PKA. Western blot analysis of HeLa cell extracts with an anti-HA antibody shows that neither PKAβ nor Pit-1 or Pit-1β alter the levels of HA-Oct-1 expression (Fig. 5C). Thus, Oct-1 is able to functionally interact with Pit-1 and Pit-1β to reconstitute the PKA response in HeLa cells. In fact, given the lower level of Pit-1β protein expression relative to Pit-1, and the lower level of basal Oct-1:Pit-1β synergy, the equivalent levels of Oct-1:Pit-1 and Oct-1:Pit-1β synergy in the presence of PKA suggest a stronger synergy between Oct-1 and Pit-1β than between Oct-1 and Pit-1.

DISCUSSION

cAMP- and PKA-dependent signaling to the rPRL promoter is unusual both for its lack of involvement of CREB and for its requirement for Pit-1 to work through the most proximal binding site, FP I (25, 30, 31). In this paper, we show 1) that Pit-1β is a more efficient transducer than is Pit-1 of the PKA signal; 2) that in addition to the β-domain, the POU-specific and POU-homeo domain are required for optimal PKA-signaling; and 3) that Oct-1 is a PKA coactivator.

The importance of Pit-1β as a nuclear integrator of signals regulating pituitary hormone gene expression is becoming increasingly clear (15, 39). Figure 2 demonstrates that Pit-1β not only is capable of transducing the PKA signaling to the rPRL promoter, but acts more efficiently than does Pit-1 (Fig. 2A) despite lower levels of protein expression (Fig. 2B). While it might be argued that this differential effect of Pit-1β *vs.* Pit-1 might be due to the different levels of protein expressed in this study, we have previously reported that with equal levels of Pit-1β and Pit-1 protein expressed, Pit-1β clearly shows an enhanced PKA response compared with Pit-1 (15). This increased efficiency of PKA signaling is, like the other effects of the β-domain, dependent on its precise AA sequence (15). The β-domain is richer in serine and threonine residues than the surrounding region, and the threonine at site 57 of Pit-1β is possibly a protein kinase C phosphorylation site (10). Thus, the β-domain contains AA acid residues directly involved in PKA-signaling. That Pit-1 is also able to transduce the PKA signal shows that the β-domain is not absolutely required for signal transduction, but rather acts to regulate it. The differential abilities of Pit-1 and Pit-1β to mediate signaling by the Ras and PKA pathways may indicate that these two isoforms serve to integrate information from separate signaling pathways at the rPRL promoter (15).

The reason for the drop in activation at the high dose of Pit-1 in the presence of PKA is not clear. That this effect is seen only with the Pit-1 isoform, but not with the Pit-1β isoform and only in the presence of PKA, suggests that there is a "titratable" component that interacts with Pit-1 but not with Pit-1β, and that the interaction is PKA dependent, and not simply squelching.

To identify Pit-1 domains common to both Pit-1 and Pit-1β that are required for efficient PKA signaling, we examined basal transcription potency and ability to transduce PKA signaling for intact Pit-1 and internal deletion mutations of Pit-1. The β-domain insertion, which enhances PKA signaling, falls in the middle of the TAD. It is possible that the β-domain eliminates an inhibitory region while inserting a stimulatory region. This is consistent with our data, shown in Fig. 3, whereby removal of either half of the TAD may increase PKA signaling. Of note, the TAD has been implicated in transducing the dopamine repression of PKA signaling (37), suggesting that this repressive function may be inherent to the TAD. Analysis of the other internal deletion mutant Pit-1 constructs indicate that the regions required for PKA signaling, as well as basal transcription potency, map to the POU-specific and POU-homeo domains, and the carboxyl-terminal region of each may be necessary for these effects. The precise role of the TAD in PKA signaling is less clear. One interpretation of the data is that the TAD and the linker between the two POU domains may function as inhibitory domains with regard to PKA signaling (Fig. 3). That deletion of the POU-specific and POU-homeo domains eliminates transduction of the PKA signal is consistent with an absolute requirement for the DNA-binding functions of Pit-1 and with the fact that Pit-1-Oct-1 interactions are mediated through these domains (38, 40).

While Pit-1 is absolutely required for PKA stimulation of the rPRL promoter, it appears not to be a direct PKA target. Pit-1 can be phosphorylated in intact cells or *in vitro* by protein kinases (41, 42). However, two recent reports have shown that phosphorylation of Pit-1 is not essential in mediating the PKA response (32, 33). The lack of correlation between PKA or protein kinase C phosphorylation of Pit-1 and rPRL activation, as well as the PKA-independent activation of rPRL promoter, strongly suggests the involvement of a coactivator in mediating the PKA/Pit-1 activation of the rPRL promoter.

Pit-1 has been shown to functionally interact, via FP I, with several transcription factors, including TEF and Oct-1, a ubiquitous homeobox protein that is found in lactotrophs, to activate basal rPRL promoter activity (35, 36). Since the PKA effect maps to FP I, we were led to directly test the ability of TEF and Oct-1 to mediate PKA signaling to the rPRL promoter. The failure of TEF to synergize with Pit-1 or Pit-1β in the presence of PKA implies that TEF is not the Pit-1/PKA coactivator and that not all FP I-binding factors can function as such. However, the ability of Oct-1 to synergize with Pit-1 and Pit-1β, either to activate basal rPRL expression (Fig. 5B) (38) or to transduce the PKA signal (Fig. 5A), demonstrates that Oct-1 is a Pit-1/PKA coactivator. Thus, the Pit-1-Oct-1-PKA interaction is a selective one.

MATERIALS AND METHODS

Tissue Culture

Monolayer cultures of HeLa human cervical carcinoma cells were kindly provided by Dr. Kathryn Horwitz (University of Colorado Health Sciences Center). Cells were maintained in DMEM, with 10% FCS (Figs. 2 and 3) and 12.5% horse serum and 2.5% FCS (Figs. 4 and 5), and 50 μg/ml of penicillin and streptomycin. Cells were maintained at 37 C in 5% CO_2, and the medium was changed no more than 16 h before electroporation. Cells used for transfections were harvested at approximately 70–80% confluence using 0.05% trypsin and 0.5 mM EDTA.

Plasmids

The pA3 −425 rPRL Luc reporter construct contains a 498-bp fragment spanning positions −425 to +73 of the rPRL gene ligated upstream to the firefly luciferase reporter gene and downstream of three polyadenylation sites in pA3luc (20, 43–47). The plasmids coding for *Escherichia coli* β-galactosidase under the control of the human cytomegalovirus immediate early promoter (pCMVβ-gal) (48, 49) (CLONTECH, Palo Alto, CA) or the Simian virus-40 early promoter (pSVβ-gal) (21) were included in transfections to control for transfection efficiency. The pRSV-PKAβ plasmid encoding the β-isoform of the PKA catalytic subunit derived from Chinese hamster ovary (CHO) cells was kindly provided by Dr. R. A. Maurer (University of Oregon Health Science Center, Portland, OR). Plasmid pRSV-Pit-1 and pRSV-Pit-1β were generously provided by Dr. M. Karin (University of California, San Diego. CA) (4, 31). Plasmid pCGN-HA-Oct-1 was a generous gift of Dr. Winship Herr (Cold Spring Harbor, NY). Plasmid pCMV-FLAG-hTEF was a generous gift of Dr. Hunger (University of Colorado Health Sciences Center).

The vectors pRSV-HA-Pit-1 and pRSV-HA-Pit-1β, which encode N-terminal influenza HA-tagged Pit-1/Pit-1β, were constructed through PCR mutagenesis of the Pit-1 TAD. Wild-type pRSV Pit-1/β plasmids were used as substrates for PCR (50), in which an HA tag was added to the amino terminus of the Pit-1/Pit-1β TAD by its inclusion in the 5′-oligonucleotide primer. To minimize the target sequence to be submitted to PCR amplification, a *Hin*dIII-*Ppu*M I fragment encompassing nucleotides 1–337 of Pit-1 or 1–415 of Pit-1β from each PCR product was subcloned into a derivatized pGem-7Z (Promega, Madison, WI) plasmid DNA whose *Sac*I site had been converted to a *Ppu*M I site (pGem7P). Commercially synthesized deoxyoligonucleotides (Macromolecular Resources, Fort Collins, CO; and GIBCO/BRL, Grand Island, NY) contained the following sequences:

5′-TAD: AAA AAG CAA GCT TCC ATG GGG TAC CCA TAC GAT GTT CCG GAT TAC GCT AGT TGC AAC CTT TC; and

3′-TAD: GTT TGT CTG GGT GTA TC.

The presence of each introduced HA-tagged Pit-1/Pit-1β fragment in pGEM-7Z was tested by digestion with restriction enzymes and verified by Sanger sequencing using reagents and protocols obtained from a commercial kit (Sequenase; United States Biochemical Corp. Cleveland, OH), and commercially available T7 and SP6 promoter-specific primers (Promega; Madison, WI). Sequencing was done both in our laboratory and through the UCHSC Cancer Center Core facility. HA-tagged Pit-1 and Pit-1β were excised from pGem-7P by digestion with *Hin*dIII and *Ppu*M I and ligated to the unique *Hin*dIII and *Ppu*M I sites of pRSV-Pit-1 to produce pRSV-HA Pit-1 and pRSV-HA Pit-1β.

Plasmid DNAs were prepared by passage over an anion exchange column (QIAGEN, Inc., Chatsworth, CA) and quantitated by absorbance at 260 nm or on a Dynaquant fluorimeter and by comparison with DNA standards on agarose gels.

Transfections

DNA was introduced into HeLa cells by electroporation as follows: approximately 2–3 × 10^6 enzymatically dispersed cells were mixed with plasmid DNA in a sterile gene-pulse chamber and exposed to a controlled electrical field of 500 μfarads at 220 V, as described previously (24). Cells from individual transfections were then maintained in DMEM, 10% FCS, and 50 μg/ml of penicillin and streptomycin at 37 C. The nonspecific effects of the rous sarcoma virus (RSV) promoter upon transcription factor availability was controlled for by including amounts of pRSV β-globin plasmid DNA in all assays to render the total pRSV DNA concentration constant.

Luciferase Assays

Transient transfections were performed in duplicate or triplicate, in at least two separate experiments. After incubation for 48 h, cells were harvested with PBS containing 3 mM EDTA, pelleted, and resuspended in 100 mM potassium phosphate buffer (pH 7.8) 1 mM dithiothreitol. Cells were lysed by three cycles of freeze-thawing and by vortexing for 1 min between thaws. Cell debris was pelleted by centrifugation for 10 min at 10,000 × *g* at 4 C, and the supernatant was used for subsequent assays. Luciferase activity in the supernatant was assayed as previously described (21). Samples were measured in duplicate using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA). Total luciferase units were normalized to β-galactosidase activity levels driven by the internal control SV40 promoter and assayed as described (Figs. 2 and 3) (48, 49) or normalized to total protein in the extract (Figs. 4 and 5) where protein assays were performed according to the method of Bradford (51) using commercially available reagents (Bio-Rad, Richmond, CA).

Visualization of Proteins

Transient transfections were performed in duplicate. For Fig. 3, HeLa cells transfected with plasmid DNAs were harvested after 24 h incubation with PBS containing 3 mM EDTA, pelleted, and resuspended in 100 mM potassium phosphate buffer (pH 7.8)-1 mM dithiothreitol. Cells were lysed by three cycles of freeze-thawing and by vortexing for 1 min between thaws. Cell debris was pelleted by centrifugation for 10 min at 10,000 × *g* at 4 C, and the supernatant and pellet were separated. The protein content of each lysis supernatant was assayed according to the method of Lowry *et al.* (52), using commercially available reagents (Bio-Rad). Pellets were then combined with equal amounts of lysis supernatant (75 μg) and used for SDS-PAGE separation.

For Figs. 2, 4, and 5, HeLa cells transfected with plasmid DNAs were harvested after 24 h with PBS containing 3 mM EDTA, pelleted, and resuspended in a TEA-SDS solubilization buffer,(55 mM triethanolamine, 111 mM NaCl, 2.2 mM EDTA, and 0.44% SDS) (53) and a mix of protein inhibitors (leupeptin, pepstatin A, chymostatin, aprotinin, antipain, and bestatin, each at 6 ng/mL) at 4 C. Lysed extracts were passed through a 25 G needle seven times. The protein content of each extract was assayed according to the method of Lowry *et al.* (52), using commercially available reagents (Bio-Rad). Equal amounts of protein (100 μg) were used for SDS-PAGE separation.

Samples were separated on 10% SDS polyacrylamide gels and transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA). The proteins of interest were visualized with appropriate antibodies and dilutions, and enhanced chemiluminescence (ECL) media (Amersham Life Sciences, Arlington Heights, IL). To demonstrate equal loading of protein from each sample, blots were stripped with a solution containing 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 2% SDS at 50 C for 30 min, and actin was visualized with a mouse monoclonal antiactin (Boehringer-Mannheim Corp., Indianapolis, IN) diluted 1:1000 and secondary sheep antimouse horseradish peroxidase (HRP)-conjugated antibodies (Amersham Life Sciences) diluted 1:10,000.

HA-tagged Pit-1 and Pit-1β (Fig. 2D), as well as HA-tagged Oct-1 (Fig. 5), were visualized with a mouse monoclonal antiactin (Boehringer-Mannheim Corp.) diluted 1:1000 and secondary sheep antimouse HRP-conjugated antibodies (Amersham Life Sciences) diluted 1:10,000. Wild-type and internally deleted Pit-1 (Fig. 3) were visualized with a rabbit polyclonal anti-Pit-1 antibody specific for AA 36–52 and 214–230 (BAbCO, Richmond, CA) diluted 1:1000 and secondary goat antirabbit HRP-conjugated antibodies (Amersham Life Sciences) diluted 1:10,000. FLAG-tagged-TEF (Fig. 4) was visualized with mouse

monoclonal anti-FLAG M2 antibody at 10 μg/ml (Kodak Scientific Imaging Systems-IBI FLAG System, New Haven, CT) and secondary sheep antimouse HRP-conjugated antibodies (Amersham Life Sciences) diluted 1:10,000.

Acknowledgments

We thank Kelley Fantle, Nicole Manning, Deirdre Cooper-Blacketer, and Jeanette Wagner for technical assistance, and members of the Gutierrez-Hartmann laboratory for their helpful suggestions and comments. We also thank Andrew Bradford, John Tentler, Dana Manning, and Phil Zeitler for critical reading and discussions of this manuscript. Tissue culture media were prepared by the Tissue Culture Core Facility of the Colorado Cancer Center.

Received May 11, 1998. Re-revision received October 14, 1998. Accepted October 15, 1998.

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This research was supported by NIH Grant DK-37667. S.E.D. was supported by a Colorado Cancer League Postdoctoral Fellowship, National Research Service Award F32 DK-09160, and a Postdoctoral Fellowship from the Lalor Foundation.

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